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# A fast, reliable and cost-effective method to generate tumor organs for therapy screening *in vivo*

**Lorena García-Hevia, Fidel Fernández, Iñigo Casafont, Juan Carlos Villegas and Mónica L Fanarraga**

Grupo de Nanomedicina, Universidad de Cantabria-IDIVAL, Facultad de Medicina, Herrera Oria, s/n, E-39011, Santander, Cantabria Spain

**E-mail:** fanarrag@unican.es

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## Abstract

Innovative anticancer treatments continuously require tissue bioengineering models to test novel therapies. The increasing number of developments based on nanotechnology for cancer therapy or theragnostics demand simple, reliable, fast and cost-effective cancer *in vivo* models for preclinical testing. However, despite the many tumor models available, very few reproduce the complex intratumoral cell-to-cell interactions as well as the accompanying systemic whole body effects resulting of the tumor organ metabolic, hormonal or growth factor activities, all having critical implications in the success of cancer therapies. Here we describe a reliable tumor model that can be easily reproduced to generate visible solid malignant melanoma tumor organs within a defined period of 5–10 days recapitulating the tumor stroma that is essential for cancer development. These models can be easily evaluated *in vivo* or by anatomo-pathological procedures. This method provides a fast, reproducible, reliable and cost-effective way to generate solid tumors for *in vivo* therapy, drug, nanomaterial or imaging probe evaluation, diagnostic or theragnostic screening and validation.

## 1. Introduction

Pharmacological and pharmacodynamic evaluation of new chemicals or nanomaterials in cancer requires inexpensive, reproducible and easily available models of study. Anticancer drug or nanomedicine effects are currently first investigated *in vitro*, in 2D and 3D cell culture systems grown from different cancer cells. These models provide a fast, reasonably efficient, inexpensive and reproducible ways of evaluating new anti-proliferative or cytotoxic therapies. However, there is ample bibliography that demonstrates how many *in vitro* validated chemicals do not behave as expected *in vivo,* when tested in real tumors. The main reason for this failure is the fact that real tumors are not just simple disorganized masses of tumoral cells [1]. Instead, tumors behave like real organs, interacting with the body releasing growth factor into the blood stream.

Tumors have a complex cellular organization where cancer cells are just a small proportion of all cells in the organ. Tumors consist of two basic components, the proliferating neoplastic parenchymal cells and the stroma. The parenchymal tumor cells contribute to the growth and evolution of the tumor and are mostly located at the proliferating borders of solid neoplasms. The stroma includes the extracellular matrix components, the mesenchymal supporting cells, the cells of the vascular system and the cells of the immune system that infiltrate the cell mass. The stromal cells provide sustain and survival to cancer cells, actively participating in tumorigenesis and vasculogenesis. Therefore, the interactions between the parenchymal cancer cells and stromal components are critical in tumor growth and progression [1, 2].

Solid cancers are often characterized by intratumoral hypoxia and acidosis [3, 4]. Both play a fundamental role in tumor progression, resistance and are associated with a significantly increased risk of metastasis and patient mortality. Therefore, hypoxia and acidosis are highly desirable in tumor models for they are critical in every aspect of cancer biology and cancer therapy screening [3, 5].

Among the different tissue bioengineering approaches to model 3D cancer *in vitro* the most typical formats are (i) spheroids, (ii) cells cultured as multicellular aggregates or organoid cultures, and (iii) cells embedded in acellular tissue supports made of natural

or synthetic engineered matrices [6–8]. These approaches, applied in the study of pancreatic [9], prostate [10], squamous cell carcinoma [11], melanoma [12], or esophageal carcinoma [13] among many others, reproduce intratumoral hypoxia reasonably well [14].

A simple 3D multicellular culture model is the organotypic culture [15]. This system allows the *in vitro* growth of small samples of malignant tissues that replicate the original tumor cell types, at least during the initial culture steps [7]. However, as these explants grow *in vitro*, the cell population in the culture evolves and often macrophages and tumoral cells are the only two cell types well represented.

Transplantation models are areal alternative strategy for anti-cancer therapy screening. Cancer cell xenotransplantation is often performed in species such as the zebra fish or the chick embryonated eggs. However, although these systems are inexpensive and do not precise special bioethical requirements, they are quite laborious, and do not allow a sufficiently reproducible systematic study for accurate statistical evaluation of the therapy tested.

Mammalian models are more interesting from the therapeutic and pharmacological-point of view. Their physiology, body temperature, pharmacodynamics, toxicology, etc can all be reasonably extrapolated to humans. As with the above methods, there are also many alternatives, benefits and limitations for these model systems. The simplest model consists of transplantation of tumor tissues or cells into a host recipient of identical genetic background or strain. This type of transplant is known as allograft or syngeneic transplant. The main advantage of this experimental approach, typically performed in mice, is the presence of a normal host immune system that does not reject the tissue. A well-known example of this type of transplantation models is to produce melanoma tumors in C57BL/6J mice injecting B16F10 cells in intravenously. This system is interesting to induce a systemic tumorigenesis, most frequently triggering metastasis in lungs [16].

Paradoxically, the main advantage of this system is also the main disadvantage since cancer tissues and the recipient must share a common genetic background and thus, this model is limited to allograft transplants from the same species, i.e. murine tissue to murine host models, exhibiting a systematic rejection of human cancer cells.

Xenograph transplantation in mice is also possible. The host mice however, must have an impaired immune systems so the transplanted foreign cells or tissues are not rejected. These xenotransplantation techniques are typically performed in immunodepressed Athymic Nude [17–19] or also NOD SCID mice [20–23]. There are many advantages in these systems. First, (i) mice are mammalian cancer model systems, (ii) there is no rejection, (iii) and there is a tumor organ development. Moreover, these mice allow the transplantation of small cancer tissue pieces, thus permitting the amplification of a replica of the whole tumoral microenvironment, including the different cell types existing in the tumor, growing malignant cell masses that are highly representative of the properties, progression and genetic variability of the particular cancer in study. These feature also allow the development of 'avatar mice' for *ad personam* drug testing [24]. But there are also disadvantages in these two models. The acquisition and housing of these mice is very expensive, in particular that of the Athymic Nude strain, since they are highly susceptible to opportunistic viral and bacterial infections. In addition, the immunodeficiency in these mice does not mimic the real tumor micro-environmental situation. Also, tumor growth in these models is slow, requiring 3–6 weeks [25].

Here we describe a new technique to develop tumor organs in murine neonate mice that has numerous advantages, namely (i) is a mammalian system where there is (ii) no rejection, (iii) a complete tumor organ development, (iv) is fully reproducible, (v) allows therapy (drugs or nanomedicines) testing, (vi) is fast (tumors develop in 6–15 days), (vii) easy and (viii) inexpensive. Other benefits include a complete tumor organ development that allows therapeutic interventions to investigate different treatment potentials, and clinical trial monitoring.

## 2. Materials and methods

### 2.1. Ethics statement

Animal work was conducted in accordance with the Guide for the 'Care and Use of Laboratory Animals' of the Spanish Ministry of Science, Research and Innovation. All methods were approved by The Committee on the Ethics of Animal Experiments of the University of Cantabria according to current EU legislation (RD 1201/2005). Next to that, all efforts were made to minimize the suffering of the mice. All animal experimentation procedures were performed humanely, according to EU legislation following the principle of the 'Three Rs', in the use of animals.

### 2.2. Cell culturing

B16F10 murine melanoma cells (ATCC® CRL-6475™) were cultured in 100 mm diameter culture Petri dishes filled with 10 ml of Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum and antibiotics (all from Life Technologies). The cells are grown in standard cell culture conditions, 37 °C 5% $CO_2$ in a humidified incubator and re-plated every 2 days to assure cell viability. Further cell line details or culturing conditions can be obtained at the ATCC web page (http://lgcstandards-atcc.org/products/all/CRL-6475.aspx).

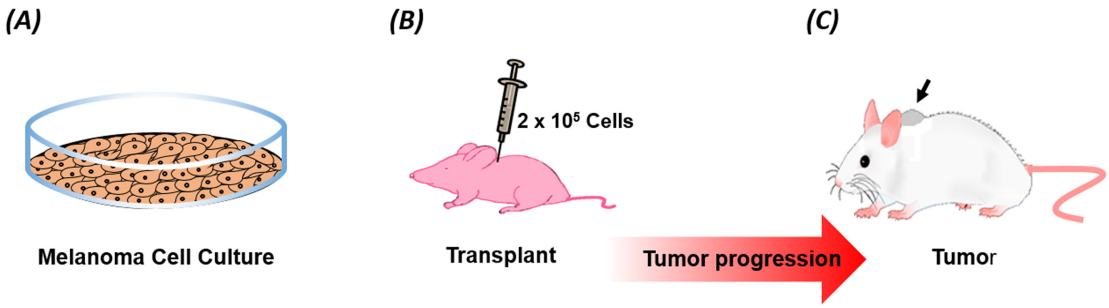


**Figure 1.** Steps in tumor organ development. (A) Murine melanoma cell line culture and amplification *in vitro*. (B) Subcutaneous interscapular transplantation of $2 \times 10^5$ melanoma cells per host. (C) Solid pigmented melanoma tumor localization and identification (arrow).

### 2.3. Experimental preparation

CD1 mice are used as hosts for cell transplantation. Whole litters bearing 10–16 pups aged 2–9 (P2–P9, see the text) were used for the experiment. Mouse pups were transplanted with a total of $2 \times 10^5$ B16F10 murine malignant melanoma cells (ATCC® CRL-6475™) in a volume of 10 $\mu$l of IMDM (Live Technologies) containing 10% serum and gentamicin subcutaneously (figure 1). Typically, a 70%–80% cell confluence Petri dish containing B16F10 melanoma cells was employed to transplant a single whole litter. The cell suspension was transitorily resuspended in 10 ml of complete tissue culture medium for cell counting in a Neubauer chamber. Cell numbers were adjusted to approximately $2 \times 10^5$ cells per ml. Aliquots of 1 ml were collected in 1.5 ml sterile centrifuge tubes that were maintained at 37 °C until the transplantation set u. is ready. Cells were finally collected by centrifugation in a volume of in 10 $\mu$l of sterile tissue culture medium containing antibiotics.

### 2.4. Sample size and transplantation procedure

The number of pups per litter significantly conditions the speed of the pup development and thus, the final tumor size. This inherent *in vivo* artifact is reduced taking each litter as an independent experiment. All littermates should be injected simultaneously following identical procedures. A total of 10 $\mu$l of melanoma cells is sterile tissue culture medium containing antibiotics is loaded in a 25–50 $\mu$l Hamilton syringe for intra-scapular subcutaneous injection.

### 2.5. Therapy testing procedure

Animal monitoring should determine when the treatment should be applied (see the text and figures). Once tumors have developed, each litter should be divided in two halves for the drug/therapy testing study, using half of the litter as a control-i.e. injected with the control excipient used for the treatment in study-, and the other half as subject of the screening or testing treatment-i.e. drugs, nanomaterials, probes, etc- (figure 2). This procedure reduces to a maximum the inherent noised of the *in vivo* screening system, reducing to a maximum the unspecific statistical variability.

### 2.6. Anatomo-pathology of the tumoral organs

Tumor growth evaluation is performed by anatomo-pathology. Mice should be sacrificed preserving the cervical region intact, for instances by anesthetic overdose. Tumor masses, localized in the interescapular region are carefully dissected. The final tumor size can be measured on the freshly dissociated tumors or fixed tissue (volume measurement or weight). Microscopic detailed analysis can be carried out in formaldehyde fixed tumors that can be routinely processed for paraffin embedding and sectioning and hematoxylin–eosin staining. Final tumor sizes can be statistically evaluated using a standard software, i.e. Sigma Plot 8.0 (Systat Software, Richmond, CA).

### 2.7. Electron microscopy

Tissue samples processed for electron microscopy were fixed with 1% glutaraldehyde in 0.12 M phosphate buffer, during 24 h, were washed in 0.12 M phosphate buffer, post-fixed in 1% buffered osmium tetroxide, dehydrated in a graded acetone series and embedded in Araldite. Semi-thin sections (1 $\mu$m) were stained with 1% toluidine blue to assess the cells. Ultrathin sections, 70 nm thick, were obtained on an LKB ultramicrotome, stained with lead citrate and uranyl acetate and observed in a Jeol 1011 microscope.

## 3. Results

### 3.1. Tumor growth monitorization *in vivo*

The under-developed immune system of these host mice together the growth factor and hormonal conditions in their tissues ensure a perfect tumoral environment to support solid pigmented tumors that develop in 4–10 days (figure 3) [26]. The developing pigmented tumor masses can be easily monitored 2–4 days post-transplantation in live animals though the skin (figure 3). Black cell masses can be very easily identified 6–8 days post-transplant. At this time treatments such

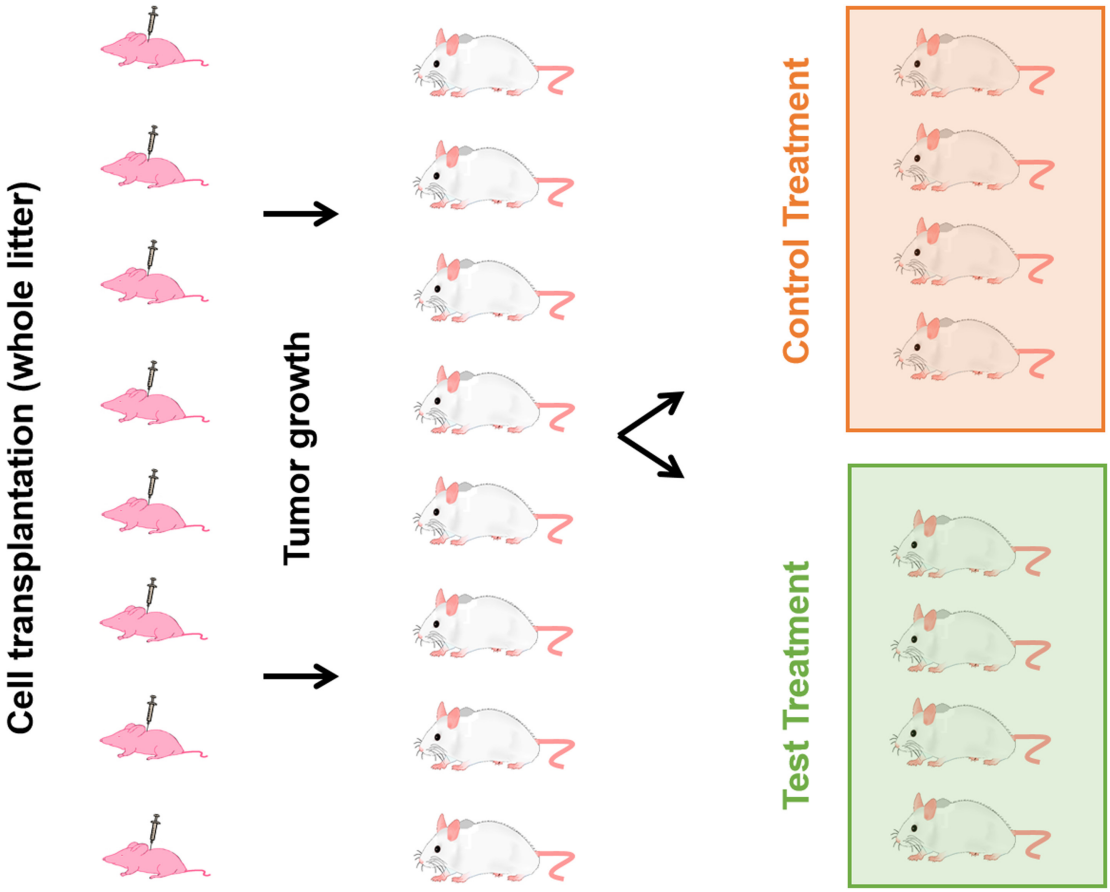


**Figure 2.** Experimental design. A whole liter is used for the experiment. All pups of the litter are transplanted following identical procedures (as described in the text). Mice bearing visible tumors are blindly divided into two groups for the treatments.


**Figure 3.** Development of the tumor organ. (A) Postnatal day 4 mice two days after transplant. Arrows point at the injection point. (B) Visible subcutaneous interescapular transplantation pigmented tumors in postnatal day 8 pups, 5 days post-transplant. Arrows indicate the localization of the tumor. This image demonstrates the high reproducibility of this tumor transplantation technique. (C) Postnatal 15 mouse bearing a solid melanoma tumor (arrow).

as intratumoral drug delivery can be easily performed. If the study requires larger tumors, solid melanoma masses can be allowed to grow for other 3–7 days (figure 3(C)). Black subcutaneous masses can be easily identified in life animals and after sacrifice for further tumor analysis (figure 4).

Tumoral organs can be allowed to grow for different time periods depending of the type of screening to be performed. However, as the host develops the immune system, the tumor growth rate diminishes, achieving a final tumoral organ size of up to 500 mg. As a general rule, host mice do not develop cachexia. Therapy can be applied 5–14 days post-transplant depending on the experimental requirements, i.e. desired tumor size at treatment, or required final tumor size. A simple day-by-day direct monitorization of the tumoral mases can be performed for in real time for therapy testing. Tumor characterization and evaluation by conventional techniques can also be performed (i.e. ultrasonography).

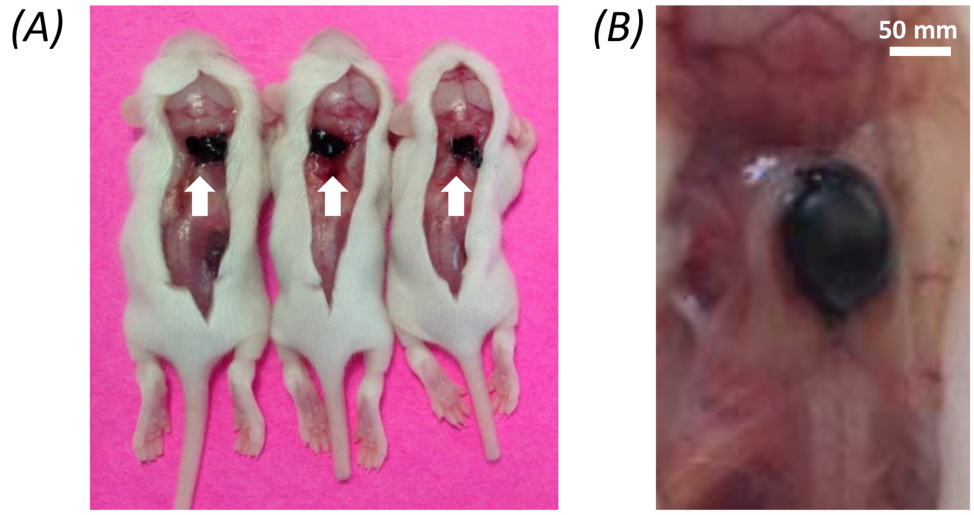


**Figure 4.** Macroscopic evaluation of the generated tumors. (A) This image shows the location and macroscopic characteristics of various melanoma tumors in 12 day old mice littermates (5 days post-transplant). Black pigmented tumors are easily identifiable for anatomo-pathological evaluation. The reproducibility of the technique is also patent. (B) Close up image of one of a melanoma tumor *in situ*. The tumor is generally encapsulated, well vascularized and can be easily dissected for further analysis (5 days post-transplant).

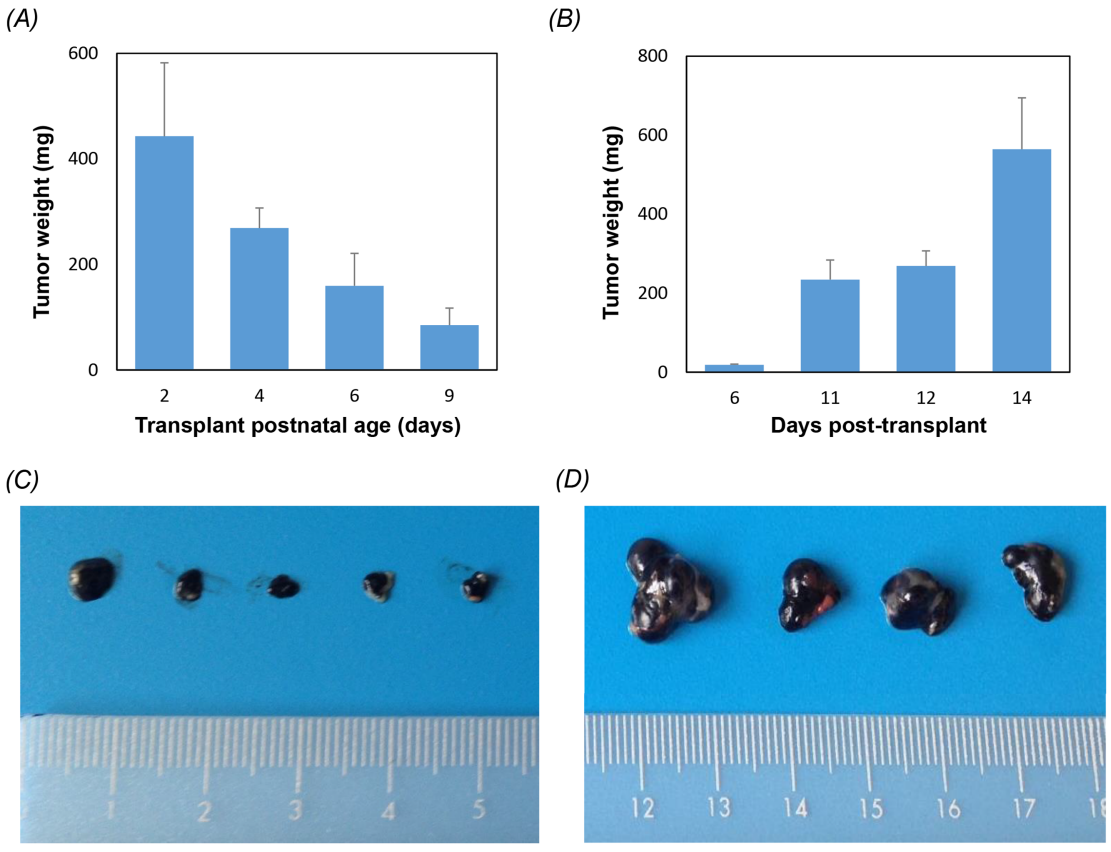


**Figure 5.** Experimental reproducibility. (A) This histogram represents the final melanoma tumor sizes in transplanted mice and the influence of host age at transplantation. Tumors were dissected 10 days post transplantation and weighed. Mice transplanted at postnatal day 2 display significantly larger tumors than mice transplanted at older ages. Notice that tumor size variability is smaller in older mice. (B) Final tumor sizes in mice transplanted a postnatal day 4 allowed to develop for 6, 11, 12 and 14 days. These results indicate that 11–12 days post-transplant enhance the experimental reproducibility. (C) Representative tumor organ growth reproducibility in postnatal day 4 transplanted mice, processed 6 days post-transplant. (D) Tumor organ reproducibility in postnatal day 4 transplanted mice, processed 14 days post-transplant.

### 3.2. Anatomo-pathology of the tumoral organs

Transplantation of melanoma cells generate very similar solid pigmented melanoma tumors in all the pups of the litter with a maximum degree of reproducibility (figure 3). Macroscopically these tumoral masses are generally well encapsulated, vascularized,

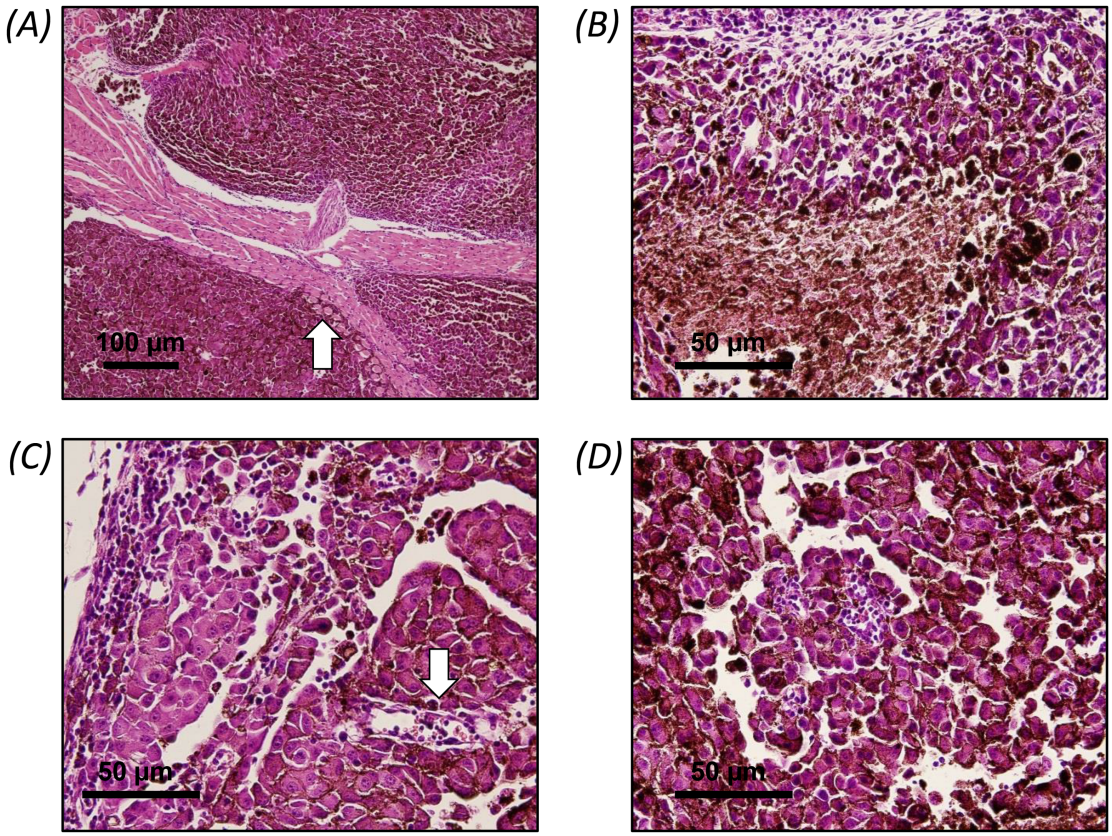


**Figure 6.** Microscopic anatomo-pathological characteristics the generated melanoma tumors. (A) Hematoxylin–eosin section of tumor displaying typical malignant tumoral features such as expansive tumor edges, and neovascularization. The transplanted cells occasionally develop infiltrating tumoral cell masses of pigmented epithelial melanoma cells (arrow). (B) Detail of the pigmented cell masses within the tumoral organ. (C) Detail of the acantholysis surrounding small blood vessels loaded with visible erythrocytes (arrow). (D) These tumors present a solid pseudo-papillary pattern and acantholysis (loss of intercellular connections). A significant inflammatory response, a high mitotic rate, intratumoral necrotic foci are also observed.

and have variable sizes depending on various key factors, namely (i) age at transplantation, and (ii) days that the tumoral mass is allowed to grow (figure 4). Tumoral organs can vary form 2–500 mm depending on these factors. Figure 5 shows the variability expected for the final tumoral masses according to these two factors. Histological examination of these tumors reveal cell masses constituted of well-organized and pigmented melanoma parenchymal cells that intermingle with multiple other cell types and extracellular matrix components (figure 6). Neovascularization of the tumor masses is also observed. These tumors display typical standard malignant melanoma aggressive features such as (i) a high mitotic rate, (ii) intratumoral necrotic foci, (iii) expansive growth edges and (iv) infiltration of surrounding tissues -namely fat, muscle and peripheral nerves-, (v) or a significant inflammatory response, among others (figures 6 and 7). Summarizing, these tumor organs are highly representative of naturally developing tumor organs and display classical hallmarks of cancer including sustained angiogenesis.

### 3.3. Versatility of the model

This tumor model system is highly versatile allowing the *in vivo* testing and validation of single treatments o repeated dosages of different therapies, from drugs to engineered nanomaterials, different pre-treatments [27, 28], diagnostic probes such as quantum dots for tumor localization or magnetic nanomaterials for diagnosis, theragnostics therapies, etc. Moreover, these therapies can be applied by all possible routes including (i) intra-venous, (ii) intra-muscular, (iii) subcutaneous, (iv) oral, (v) intra-tumoral injection or by any other parenteral route, etc.

### 3.4. Reproducibility of the model

Reproducibility of the experimental conditions is generally the major handicap of *in vivo* therapy testing. This tumor models are highly reproducible compared to most 3D tumoral models that often produce tumor cell masses are not directly comparable in different individuals, mostly due to experimental artifacts resulting from complex technical approaches. This experimental variability does not allow to discriminate the effect of the therapy tested from that of the being investigated compared to that of the placebo or control. Figures 3–5 show the high degree of reproducibility of the system reducing to a minimum the inherent noise of these type of transplantation experiments.

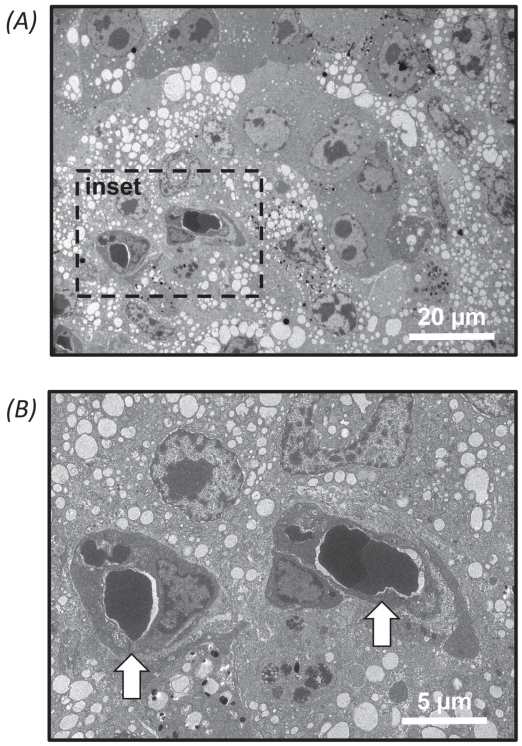


**Figure 7.** Electron micrograph of a melanoma tumor. (A) Melanoma cells develop a rich tumoral microenvironment where parenchymal cells intermingle with fat cells, macrophages and abundant neovasculature. (B) Detail of the intratumoral blood vessels. Arrows point at red blood cell sections visible within neo-capillaries.

## 4. Discussion

The urgent need for the development of simple, cost-effective and physiologically relevant models in cancer clinical trials has forced to develop many different experimental systems to reproduce tumoral conditions. As a first approach, preclinical drug testing typically performed *in vitro* is likely to produce meaningful results. However, as the assays and questions become more refined, tissue culture models cannot recapitulate many aspects of human cancers including the genesis, progression, and clinical course. Hence, *in vivo* cancer models displaying different degrees of sophistication must be used to test cancer therapeutics or diagnostic probes. Among these, the murine model is the most widely used tool.

There are many murine models currently available that replicate countless tumoral characteristics. At the present time, there are murine cancer models that can be developed ad hoc, on the basis of particular clinical trial or studies. Among these there are (i) genetic transgenic germline models, (ii) gene knock-outs and knock-ins, (iii) conditional or inducible cancer systems, (iv) models of insertional mutagenesis, (v) RNA interference cancer models, (vi) viral-mediated mutagenesis, (vii) treatment with chemical carcinogens, (viii) murine chimeras, etc. However, genetic mouse models are far too sophisticated, difficult to produce, expensive and take months or years to developed, and thus result inaccessible for many laboratories.

The transplantation models discussed in the introduction of this manuscripts are more versatile for routine assays and, compared to genetic models, are low-cost for general therapy testing. However, while most of these systems can provide meaningful results for many drug or therapy aspects, most of these experimental models lack the degree of reproducibility necessary to perform a reliable statistical analyses. This study provides a new alternative model for preclinical drug testing *in vivo* that is reliable, cost-effective, reproducible and only takes a few days for the experimental setup to generate tumor organs for therapy screening *in vivo*.

## 5. Conclusion

The development of new therapies requires simple but reliable models for screening, ideally mammalian 3D multicellular models that can be easily monitored by untrained scientists to allow a fast preliminary evaluation of new compounds. Most mammalian *in vivo* systems require high tech skills that are often inexistent in most routine laboratories. Here we describe a mouse cell transplantation model for the study of cancer therapies that can be monitored by naked eye, can be treated with any putative therapy by any route, is fully reproducible and thus, is a highly convenient for most routine preclinical therapy testing assays, making *in vivo* therapy evaluation simple and efficient.

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